THE MEASUREMENT OF TISSUE ACID MUCOPOLYSACCHARIDES [1]

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Investigation of the metabolism of connective tissue acid mucopolysaccharides in normal and abnormal states has been hampered by a lack of practical analytical methods. Reported methods for measuring tissue acid mucopolysaccharides involve multiple steps of enzyme digestion and precipitation (1, 2), with consequent risk of loss of material as well as poor adaptability for multiple analyses. This report describes a less complex procedure which gives reproducible results and good recovery of test material. Values are given for the acid mucopolysaccharide content of human dermis, subcutaneous tissue, aorta and thyroid gland, using both orcinol and carbazole reactions for uronic acid. Chromatographic and electrophoretic studies of the polysaccharides found and determination of the proportion digested by testicular hyaluronidase are also reported.

METHODS

Defatting and drying. Fresh or frozen tissue samples of 2 grams or less, cut into pieces about 2 mm. in diameter, were placed in 100 ml. of acetone on a wrist-action mechanical shaker for one hour. This was followed by additional one hour extractions with two 50 ml. quantities of acetone, and two 50 ml. quantities of diethyl ether. The tissue was then lyophilized overnight and stored in a desiccator.

Determination of total acid mucopolysaccharide content. 1. Weighed aliquots (75 to 200 mg.) of dried defatted tissue were extracted in 50 ml. of 0.5 N NaOH in a Waring blendor for 45 minutes in a cold room. The extraction procedure was stopped every 10 minutes to allow cooling of the container in an ice bath. The residual tissue was removed by centrifugation.

2. Ten ml. aliquots of the extract were placed in centrifuge tubes and brought to near neutrality by the addition of 0.5 ml. of 8.75 M acetic acid to each.

3. Three ml. of 17 per cent perchloric acid was then added to each tube to precipitate protein. After refrigeration at 4° C. for one hour to allow the precipitate to flocculate, the tubes were centrifuged at 2,000 rpm for 20 minutes.

4. Ten ml. of each supernatant solution was dialyzed overnight against running cold tap water to remove the perchloric acid and salts.

5. The dialysis bags were emptied quantitatively and washed with water; 0.2 ml. of 1 per cent protamine sulfate was then added to each. After refrigeration for one-half hour at 4° C., they were spun in a Servall SS-1 angle centrifuge at approximately 3,000 rpm for 30 minutes.

6. The precipitate was dissolved in 2.2 ml. of 2 M acetate buffer of pH 5.0 and duplicate 1.0 ml. aliquots were taken for determination of uronic acid by the carbazole method of Dische (3), with slight modification as previously reported (4).

7. Using orcinol, a combined uronic acid and hexose determination was done on a second 10 ml. aliquot of each original extract, processed in similar fashion up to step 6, and dissolved in the same buffer. The procedure of Brown (5) was followed; optical densities were read at 520 mμ and 670 mμ. An equimolar mixture of galactose and mannose was used as standard solution in addition to glucuronolactone, and calculations were done algebraically, based on the amount of color contributed by each type of sugar at the two wave lengths read.

Effect of incubation with testicular hyaluronidase. A 25 ml. aliquot of each original NaOH extract, in a 40 ml. centrifuge tube, was brought to near neutrality by the addition of 1.3 ml. of 8.75 M acetic acid. Proteins were removed by centrifugation at approximately 2,000 rpm for 20 minutes after the addition of 1.7 ml. of 60 per cent perchloric acid, and refrigeration at 4° C. for 30 minutes. Twenty-five ml. of the supernatant solution was dialyzed overnight against running cold tap water. The solution was transferred quantitatively and the volume brought to exactly 31 ml. with water. Two aliquots of 10 ml. were taken, and buffer was added to a final concentration of 1.0 M acetate, pH 6.0, and 0.15 M NaCl. Approximately 30 turbidity reducing units (TRU) [3] of commercial testicular hyaluronidase (Worthington or General Biochemical) was added to each. After incubation for 15 hours at 37° C., another 30 TRU of testicu-

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[3] Assayed by the method of DiFerrante (6), which gave considerably lower values than the standard turbidimetric method of Meyer (7), run simultaneously by Dr. Manuel Epstein of the Endocrine Laboratory of Wayne State University College of Medicine.

lar hyaluronidase was added and incubation was continued for 5 hours. One ml. of 60 per cent perchloric acid was then added to remove the hyaluronidase protein. After centrifugation for 20 minutes at 2,000 rpm, 12 ml. aliquots were dialyzed overnight against running cold tap water. Dialysis bags were then emptied quantitatively and 0.2 ml. of 1 per cent protamine sulfate was added. After refrigeration, carbazole and orcinol determinations were performed.[4]

Hexosamine determinations. These were done following hydrolysis in 2 N HCl at 100° C. for 15 hours; the HCl was then rapidly evaculated with a vacuum pump. The method of Boas (8) was then followed.

Chromatographic and electrophoretic studies. Aliquots of the 0.5 N NaOH extract were processed through step 4 as above; the dialyzed solutions were then lyophilized to dryness. The residue was dissolved in a small quantity of water and chromatographed on S and S 598 filter paper, descending, using the buffered ethanol and propanol solvent systems and staining procedure previously reported (4). Electrophoresis was performed in a horizontal vapor-chamber apparatus, using 0.05 M acetate buffer of pH 4.2 at 400 volts for 3 hours. Papers were oven dried, fixed and stained in the same fashion as the chromatograms. Standards used included chondroitin sulfate from bovine nasal cartilage, hyaluronate from human synovial fluid and cock's comb, and heparin from commercial sources.

RESULTS

Completeness of extraction of acid mucopolysaccharides, as estimated by determination of hexosamine remaining in the tissue fragments after NaOH treatment, exceeded 95 per cent.

Recovery of chondroitin sulfate added to 50 ml. of 0.5 N NaOH in a Waring blendor and carried through the entire method averaged 92.1 per cent (Table I). When the chondroitin sulfate was exposed to the NaOH for longer than one hour, recoveries tended to be lower. Acetic acid was added, therefore, as soon as the extraction could be considered complete. A drop of phenolphthalein was useful to insure that the pH of the solution was kept on the alkaline side of neutrality, to prevent precipitation of mucopolysaccharide.

[4] Following completion of the studies reported herein, it was found that the method could be further simplified without lowering recoveries or reproducibility. Two ml. of 8.75 N acetic acid and 3 ml. of 70 per cent perchloric acid were added to the entire 50 ml. of extract before removal from the Waring blendor. After 30 minutes at 4° C., the tissue residue and precipitate were removed by centrifugation. The supernatant solution was divided into 4 aliquots of 10 ml., which were dialyzed and then processed as described above from step 5.

TABLE I

Recovery of chondroitin sulfate added to 50 ml. of 0.5 N NaOH and carried through entire procedure

Amount added (as uronic acid)	Recovered
μg.	%
266	103
287	91.3
287	78.8
288	79.3
292	87.5
292	111
292	93.1
307	94.8
307	86.9
310	94.2
310	89.3
310	96.2
Average	92.1

Reproducibility. Repeated determinations on the same tissue gave variations of approximately plus or minus 7 per cent for the carbazole and orcinol determinations of the total acid mucopolysaccharide. After hyaluronidase incubation, a variation of approximately plus or minus 8 per cent was found.

Interference by hexose-containing compounds. A small quantity of hexose, usually around 5 μg., was found in the final protamine precipitates by the orcinol determination, indicating the absence of significant contamination with glycogen or glycoprotein. The optical density produced by hexose in each carbazole determination was rarely more than 5 per cent of the total, and never more than 10 per cent. Correction for this contamination was not considered advisable. The absorption spectrum of the final precipitate in the carbazole determination matched that of glucuronolactone.

Effects of hyaluronidase incubation. Incubation with hyaluronidase removed an average of 88 per cent of chondroitin sulfate introduced in amounts of 20 to 3,500 μg. under the conditions tested; 74 per cent or more was removed in 95 per cent of the tests. When smaller amounts of chondroitin sulfate were used as starting material, digestion was not as complete, presumably because of the very low substrate concentration. In the studies reported, more than 20 μg. of acid mucopolysaccharide was present in the actual incubation mixture, but not all the mucopolysaccharide present was hyaluronidase-labile. In addition, some of the tissue extracts might have

TABLE II

Acid mucopolysaccharide content of samples of human dermis *

Age	Sex	Total acid mucopolysaccharide			Mucopolysaccharide remaining after hyaluronidase digestion		
		Carbazole	Orcinol	Carbazole/ orcinol	Carbazole	Orcinol	Carbazole/ orcinol
48	M	64.6	80.4	0.80	47	80	0.59
51	F	89.2	102	0.87	35	72	0.49
65	M	90.0	145	0.62	38	97	0.39
70	F	102	110	0.93	38	68	0.56
71	M	69.4	102	0.68	35	78	0.45
79	M	111	140	0.79	35	87	0.63
82	F	47.6	76.1	0.63	41	74	0.55
		Average		0.76	Average		0.52

* Figures given are μg. of uronic acid in acid mucopolysaccharide per 100 mg. of dried, fat-free tissue found using carbazole and orcinol determinations. Two hundred mg. samples were used for extraction.

contained material with an inhibitory effect on the activity of the enzyme. The results reported can therefore be considered only an estimate of the minimal amount of mucopolysaccharide labile to testicular hyaluronidase.

Samples of chondroitin sulfate were digested with hyaluronidase according to the procedure described and then dialyzed against tap water. Carbazole determinations showed that over 85 per cent of the original material had been reduced to dialyzable units.

Acid mucopolysaccharide content of human tissues. Tissues were obtained at autopsy up to 15 hours after death. Skin and subcutaneous tissue were obtained from the upper thoracic region when there had been no recognizable disease involving that area. Subcutaneous tissue was carefully separated by sharp dissection and the epidermis was removed by scraping with a razor blade. The acid mucopolysaccharide content of samples of dermis are given in Table II, and of samples of subcutaneous tissue in Table III.

Segments of lower thoracic aorta were obtained which were free of more than minimal gross atherosclerosis. The acid mucopolysaccharide content of these aortas are reported in Table IV.

Thyroid glands were obtained at autopsy and following surgical removal. The acid mucopolysaccharide levels obtained are reported in Table V.

Qualitative studies. To establish the mucopolysaccharide nature of the uronic acid found, the ratio of hexosamine to uronic acid (carbazole method) in the final protamine precipitate was determined for each tissue. Almost all of the ratios fell between 0.7 and 1.2, averaging approximately one for each of the tissues studied.

Between one-third and two-thirds of the acid mucopolysaccharide present in the extracts of the

TABLE III

Acid mucopolysaccharide content of samples of human subcutaneous tissue *

Age	Sex	Total acid mucopolysaccharide			Mucopolysaccharide remaining after hyaluronidase digestion		
		Carbazole	Orcinol	Carbazole/ orcinol	Carbazole	Orcinol	Carbazole/ orcinol
48	M	86.4	111	0.78	52	64	0.82
49	F	106	128	0.83	87	93	0.93
54	M	105	129	0.81	48	70	0.68
65	M	98.9	124	0.80	66	78	0.84
70	F	81.0	144	0.56	62	83	0.74
79	M	145	153	0.95	73	88	0.83
		Average		0.79	Average		0.81

* Figures given are μg. of uronic acid in acid mucopolysaccharide per 100 mg. of dried, fat-free tissue found using carbazole and orcinol determinations. Two hundred mg. samples were used for extraction.

TABLE IV

Acid mucopolysaccharide content of human aorta *

Age	Sex	Total acid mucopolysaccharide			Mucopolysaccharide remaining after hyaluronidase digestion		
		Carbazole	Orcinol	Carbazole orcinol	Carbazole	Orcinol	Carbazole/ orcinol
39	F	792	505	1.6	248	197	1.3
39	M	612	425	1.4	231	178	1.3
41	M	414	283	1.5	199	147	1.4
46	M	573	350	1.6	239	189	1.3
54	M	625	477	1.3	262	224	1.2
		Average		1.5	Average		1.3

* Figures given are μg. of uronic acid in acid mucopolysaccharide per 100 mg. of dried, fat-free tissue found using carbazole and orcinol determinations. Seventy-five mg. samples were used for extraction.

tissues studied was found to be digested by testicular hyaluronidase (Tables II and through V).

Electrophoresis of extracts of the four tissues studied revealed two components which stained metachromatically with toluidine blue. The faster component had the same mobility as control samples of chondroitin sulfate and heparin; these compounds were not separated by this technique. The slower component had the same mobility as control samples of hyaluronate.

In order to determine whether two or three components were present, pyridine and ammonium sulfate were used to precipitate the hyaluronate-like component in each extract, following the method of Jeanloz and Forchielli (9). The pellicle which separated at the interface had the chromatographic characteristics of hyaluronate. The aqueous phase was found to be free of hyaluronate-like material in the propanol system (Table VI, A), but still contained two components in the ethanol system. One of the components remaining in the aqueous phase had the mobility of heparin and the other moved like chondroitin sulfate (Table VI, B).

DISCUSSION

The method described permits a reasonably reliable determination of the quantity of acid mucopolysaccharide present in tissue samples. The levels found are significantly higher than those previously reported for skin (1) and aorta (2). The ratio of hexosamine to uronic acid in the final precipitate obtained from the extracts of all four tissues was usually close to the theoretical ratio of 1.0, establishing that the material found is mucopolysaccharide and of sufficient purity for quantitative studies. Additional evidence of the nature of the material measured included the digestion of a portion of the extract of each tissue by testicular hyaluronidase, the paper chromatographic and electrophoretic mobilities of the compounds present, and their metachromatic staining with toluidine blue.

TABLE V

Acid mucopolysaccharide content of human thyroid tissue *

Diagnosis	Total acid mucopolysaccharide			Mucopolysaccharide remaining after hyaluronidase digestion		
	Carbazole	Orcinol	Carbazole/ orcinol	Carbazole	Orcinol	Carbazole/ orcinol
Normal	127	110	1.2	33	46	0.73
Normal	64.5	93	0.69	20	28	0.73
Colloidal adenoma	151	114	1.3	77	88	0.87
Colloidal adenoma	156	204	0.76	58	70	0.83
Colloidal adenoma	139	173	0.80	75	107	0.70

* Figures given are μg. of uronic acid in acid mucopolysaccharide per 100 mg. of dried, fat-free tissue found using carbazole and orcinol determinations. One hundred fifty mg. samples were used for extraction. Normal thyroids were obtained at autopsy, others by surgical removal.

TABLE VI

Results of paper chromatography of extracts of tissues *

	Chondroitin sulfate	Heparin	Hyaluronate	Whole extract	Pyridine pellicle	Aqueous phase
A.	Chromatography in buffered propanol					
	0.9	0.8	0.1	0.1 and 0.8	0.1	0.8 only
B.	Chromatography in buffered ethanol					
	0.8	0.1	0.1	0.1 and 0.8		0.1 and 0.8

* Pyridine pellicle refers to the material which separated at the interface when the whole extract in water was treated with pyridine and ammonium sulfate, as described. Aqueous phase refers to the material remaining after removal of the pellicle. Figures given are Rf values at 25° C. for each component observed.

At least one mucopolysaccharide, chondroitin sulfate B, contains iduronic acid. Iduronic acid, compared with glucuronic acid, gives considerably less color in the carbazole determination but slightly more color in the orcinol determination (10). The ratio of carbazole to orcinol values for a sample of chondroitin sulfate B was found to be 0.5, which is similar to previous reports (11). Since glucuronic acid was the standard used in all determinations, the presence of chondroitin sulfate B in tissue extracts in sufficient quantity would produce carbazole to orcinol ratios of less than one. The ratio would be lowered by incubation with hyaluronidase, since material with a higher ratio would be digested leaving the hyaluronidase-resistant chondroitin sulfate B in higher relative concentration. Chondroitin sulfate B has been reported to be the major mucopolysaccharide component of pigskin and bullhide (12). The finding of an average ratio of 0.76, falling to 0.52 after hyaluronidase treatment, indicates the presence of chondroitin sulfate B as a major component of the acid mucopolysaccharide in human dermis. The carbazole to orcinol ratio of 1.5 found in aorta may be due to the presence of compounds such as heparitin sulfate, recently described by Meyer, Hoffman, and Linker, and found to be present in aorta in considerable concentration. This compound was reported to have a carbazole to orcinol ratio around 2.0 (13).

The presence of acid mucopolysaccharide in thyroid tissue has been suggested by its very high hexosamine content (8), histochemical observations (14), and the finding of a fall in the viscosity of colloid following exposure to hyaluronidase (15). Boas and Foley (16) subjected extracts of thyroid tissue to electrophoretic analysis and could account for all the hexosamine on the basis of the proteins present; no uronic acid in mucopolysaccharide could be found by their methods. By our procedure acid mucopolysaccharide was demonstrated to be present in thyroid tissue. Although it contained only one per cent of the total hexosamine, the average concentration of acid mucopolysaccharide was found to be higher than in subcutaneous tissue or dermis.

The chromatographic and electrophoretic characteristic of the three components found cannot be considered to establish their identity. This is particularly notable in the case of heparin, which was used as a standard for reasons of economy; chondroitin sulfate B was found to have the same mobility characteristics in the solvent systems used (4). It is possible that other mucopolysaccharides have characteristics similar to the standards used in this study.

SUMMARY

1. A method is described which permits the determination of the total amount of acid mucopolysaccharide in tissue samples, and gives some information concerning the nature of the mucopolysaccharides present.

2. The total amount of acid mucopolysaccharide present in human dermis, subcutaneous tissue, aorta and thyroid tissue is reported.

3. Electrophoretic and chromatographic studies revealed the presence of metachromatic staining compounds in extracts of each tissue. These were resolved into three components with mobility characteristics resembling control preparations of chondroitin sulfate A, heparin and hyaluronate.

4. Comparison of carbazole and orcinol values for uronic acid indicates the presence of chon-

droitin sulfate B as a major component of the acid mucopolysaccharide in dermis.

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